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The *Drosophila* FMRP and LARK RNA-binding proteins function together to regulate eye development and circadian behavior

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Abstract

Fragile X Syndrome (FXS) is the most common form of hereditary mental retardation. FXS patients have a deficit for the Fragile X Mental Retardation Protein (FMRP) that results in abnormal neuronal dendritic spine morphology and behavioral phenotypes including sleep abnormalities. In a *Drosophila* model of FXS, flies lacking the dfmr1 protein (dFMRP) have abnormal circadian rhythms apparently due to altered clock output. In this study, we present biochemical and genetic evidence that dFMRP interacts with a known clock output component, the LARK RNA-binding protein. Our studies demonstrate physical interactions between dFMRP and LARK, that the two proteins are present in a complex *in vivo*, and that LARK promotes the stability of dFMRP. Furthermore, we show genetic interactions between the corresponding genes indicating that dFMRP and LARK function together to regulate eye development and circadian behavior.

INTRODUCTION

Fragile X Syndrome (FXS) (O'Donnell and Warren, 2002) is associated with sleep disorders, autism, hyperactivity and cognitive deficits (Gould *et al.*, 2000; Bakker and Oostra, 2003; Miano *et al.*, 2008). FXS typically results from a CGG trinucleotide repeat expansion in the 5' untranslated region of *FMR1* resulting in loss of the *FMR1* product, FMRP (O'Donnell and Warren, 2002). FMRP and its autosomal paralogs, FXR1P and FXR2P make up a small family of RNA-binding proteins containing two KH domains and an RGG region (O'Donnell and Warren, 2002). FMRP associates with translating polyribosomes in an RNA-dependent manner (Khandjian *et al.*, 1996; Feng *et al.*, 1997a) and appears to function in translational control (Feng *et al.*, 1997b; Morales *et al.*, 2002; Jin and Warren, 2003). Putative targets of FMRP have been identified in mammals and *Drosophila* using biochemical and genetic approaches (Brown *et al.*, 2001; Darnell *et al.*, 2001; Reeve *et al.*, 2005; Zarnescu *et al.*, 2005).

Insights into FMRP function have been gained from *Drosophila* and mouse models of FXS. FMRP participates in signaling through metabotropic glutamate receptors (mGluR) and limits protein-dependent long-term depression (LTD) in the hippocampus and cerebellum, a process responsible for activity-guided synapse removal (Huber *et al.*, 2002; Bear *et al.*, 2004). *Fmr1* knockout mice have exaggerated LTD, which could contribute to cognitive deficits (Huber *et al.*, 2002; Bear *et al.*, 2004; Koekkoek *et al.*, 2005). These knockout mice also display

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phenotypes reminiscent of those observed in human patients such as dendritic spine abnormalities and learning deficits (Greenough *et al.*, 2001; Bakker and Oostra, 2003; Koekkoek *et al.*, 2005).

Loss of function of the single homolog of *FMR1* in *Drosophila*, *dfmr1*, results in viable flies (Zhang *et al.*, 2001). The *Drosophila* protein, dFMRP behaves as a translational repressor (Laggerbauer *et al.*, 2001; Li *et al.*, 2001; Zhang *et al.*, 2001; Reeve *et al.*, 2005), and RNA targets have been identified using biochemical and genetic approaches (Reeve *et al.*, 2005; Zarnescu *et al.*, 2005). Importantly, flies lacking FMRP exhibit neuroanatomical and behavioral phenotypes that include defective neurite extension, courtship, learning, and circadian clock output (Dockendorff *et al.*, 2002; Inoue *et al.*, 2002; Morales *et al.*, 2002; Pan *et al.*, 2004). Administration of lithium or metabotropic glutamate receptor (mGluR) antagonists can rescue the neuroanatomical, courtship and learning, but not the circadian phenotypes (McBride *et al.*, 2005). These studies indicate that in *Drosophila*, as in the mouse, mGluR signaling may contribute to the altered behavior.

Another clock output component, LARK, is also an RNA-binding protein and a member of the RNA Recognition Motif (RRM) class of proteins (Newby and Jackson, 1996). Previous studies have shown that LARK displays circadian oscillations in abundance in flies and mammals (McNeil *et al.*, 1998; Kojima *et al.*, 2007); in flies, overexpression of LARK in Timeless (TIM)- or Pigment Dispersing Factor (PDF)-containing clock cells causes arrhythmic locomotor activity without affecting the molecular oscillator (Schroeder *et al.*, 2003). These results are indicative of a role for LARK in clock output. While LARK has a pan-neuronal and nuclear pattern of localization (Zhang *et al.*, 2000), the mammalian homolog (mLARK or RBM4) has the capacity to shuttle into the cytoplasm (Lai *et al.*, 2003) suggesting a possible role in translational control.

We report physical and functional interactions between LARK and dFMRP *in vivo*, and suggest that the two RNA-binding proteins may cooperate to regulate the translation of certain common target RNAs.

EXPERIMENTAL PROCEDURES

Immunoprecipitation and Immunostaining Techniques

Adult fly heads were collected and homogenized in 1 ml ice-cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 30 mM EDTA, 0.5% Triton X-100) with 2X complete protease inhibitors. All further manipulations of the head lysates were performed at 4ºC or on ice. Debris was pelleted and the supernatant was collected and pre-cleared for 1 h with 100ul recombinant protein G agarose (Roche). Anti-dFMRP or anti-LARK antibody (see source below) was incubated with recombinant protein G agarose at 4ºC for 2h. The precleared lysates were immunoprecipitated with antibody-coated recombinant protein G agarose at 4ºC overnight. The precipitated complexes were used for western blotting. Anti-dFMRP antibody was used for western blotting at a dilution of 1:500 and anti-LARK at a dilution of 1:20000.

Whole mounts of the adult fly brain were immunostained according to published methods (Benito *et al.*, 2007). Antisera against PDF (anti-PAP from Paul Taghert, Wash. U.), dFMRP (monoclonal 5B6 from Univ. Iowa Hybridoma Center) and LARK (McNeil *et al.*, 2004) were employed at dilutions of 1/1000, 1/100 and 1/100, respectively.

Drosophila Genetics

Transgenic flies carrying the *UAS-ds-lark* (*lark RNAiA*) construct were generated using standard methods. The $dfmr1^{3}$ loss of function allele used in the experiments was a kind gift from Dr. Tom Jongens U. Pennsylvania). All other lines used in this study were obtained from

the Bloomington *Drosophila* stock center. Fly strains and genetic crosses were reared on standard medium with added yeast paste at 25°C.

Bioinformatics

To identify potential LARK/FMRP common targets, we searched the published mRNA target lists for both proteins (Zarnescu *et al.*, 2005; Huang *et al.*, 2007). The expression data reported in Zarnesu *et al.* were filtered to contain only those probesets that have the Affymetrix "Present" call. The average value of expression was calculated for each sample condition present in the expression data. The fold change for Fmrp IP *vs*. input mRNA was calculated for both the wild type (WT) and the mutant. Probesets showing a WT fold change of 1.4 and enriched by at least 2 fold compared to the mutant fold change were selected. The FBgn IDs of these probesets were matched to the 216 known LARK target FBgn IDs to identify common targets.

Behavioral Analysis

Locomotor activity was monitored in single flies using monitors from Trikinetics as previously described (Levine *et al.*, 1994). Activity records were visualized and statistically analyzed using a MatLab-based package known as Fly Toolbox (Levine *et al.*, 2002). Individuals were considered rhythmic if there was statistically-significant evidence of periodicity using the Fly toolbox package. The robustness of rhythmicity was assessed using the Rhythmicity Index (RI) of Levine *et al.*(Levine *et al.*, 2002). For behavioral analysis, flies were maintained in LD 12:12 at 25°C for approximately 6 days and then transferred to DD, for approximately 10 days.

Scanning Electron Microscopy (SEM)

SEM was performed using whole flies that were dehydrated in ethanol prior to microscopy.

RT-PCR

RNA samples were prepared from third-instar larvae (*y w* and *lark* null) using Trizol (Gibco BRL). RNA was reverse-transcribed with oligo$(dT)_{12\text{-}18}$ and SuperScript II (Invitrogen). PCR reactions were carried out using dFMRP specific primers.

RESULTS

dFMRP and LARK are present in a complex *in vivo*

We carried out a yeast two hybrid screen to identify proteins in a *Drosophila* library that interact with dFMRP. Such interacting proteins might provide additional insights about the cellular and biological functions of dFMRP. We employed 4 different *dfmr1* constructs as bait against a fly library. These baits expressed the KH domains of the protein (60-1), the first 500 bp of N-terminal coding sequence (60-2), the N-terminal half of the protein (60-3), or the C-terminal half of the protein (60-4). We found that bait 60-3, which contains the FMR1/FXR interaction domain, bound to approximately thirty different proteins, consistent with the known role of the dFMRP N-terminus in mediating protein-protein interactions (Reeve et al., 2008). One of these proteins, detected twice in our experiments, was *Drosophila* LARK (Supplemental Figure 1).

To verify that LARK and dFMRP are found in a complex *in vivo*, we attempted to co-immunoprecipitate the two proteins from protein extracts of adult fly heads using anti-LARK and anti-dFMRP antibodies. In two independent experiments, anti-dFMRP precipitated dFMRP and a small amount of LARK whereas anti-LARK brought down LARK and a small amount of dFMRP (Figure 1A). Neither protein was precipitated in negative control experiments (beads alone and no antibody; data not shown). The non-stoichiometric ratio of the proteins in these co-IP experiments may reflect the steady-state localization patterns of the

two proteins (LARK in the nucleus, dFMRP in the cytoplasm) (Wan *et al*., 2000; Zhang *et al.*, 2000; Morales *et al.*, 2002). We postulate that the two proteins may interact only transiently when one of the two proteins shuttles to the other intracellular compartment. Studies of mLARK and FMRP shuttling behavior are consistent with this idea (Feng *et al*., 1997b; Lai *et al*., 2003).

dFMRP levels are reduced in larval extracts concomitant with decreased LARK

In the course of pursuing co-immunoprecipitation experiments, we examined LARK abundance in *dfmr1* mutants and dFMRP levels in larvae lacking LARK protein (null $lark^{1}$ mutants survive until early pupal stages) or expressing a *lark* RNA interference (*lark RNAiA*) transgene that knocks down but does not eliminate LARK protein (Sundram and Jackson, unpublished results). Whereas *dfmr1* mutants had normal levels of LARK (data not shown), we were surprised to discover that larvae lacking LARK and adults with reduced abundance for the protein had significantly decreased dFMRP levels (Figure 1B, lanes 2, 4 and 5), relative to controls. Interestingly, *dfmr1* RNA abundance appears to be normal in lark null larvae (Figure 1C**),** indicating that LARK post-transcriptionally regulates dFMRP, perhaps by controlling the translation of *dfmr1* RNA or by stabilizing dFMRP protein.

Genetic interactions between *dfmr1* and *lark*

We employed two different phenotypic assays, eye morphology and circadian activity, to determine if *dfmr1* and *lark* genetically interact *in vivo*. As both mutations alter circadian locomotor activity rhythms, we examined this behavior in different *dfmr1*/*lark* genotypes.

Over-expression of LARK, using an eye-specific driver (GMR-Gal4), leads to a rough eye phenotype in which ommatidia are fused and there is disorganization of inter-ommatidial bristles (Figure 2B). Whereas *dfmr1* null flies have wild-type eye morphology (Figure 2A), flies simultaneously lacking dFMRP and over-expressing LARK have a more severe eye phenotype than control flies over-expressing LARK alone (compare Figure 2B to C). Figure 2 shows this interaction using the $dfmr1^{3}$ allele, but similar results were obtained using $dfmr1^{113}$ (data not shown). These results indicate that eliminating dFMRP enhances the LARK-induced eye phenotype, and suggests that *dfmr1* dosage is important for LARK function. To validate the genetic interaction between *dfmr1* and *lark* in a more biologically relevant assay, we investigated the effects of altering the levels of dFMRP and LARK on circadian rhythms.

It has previously been shown that GAL4-driven overexpression of LARK within pacemaker cells results in arrhythmic locomotor activity for many flies (Schroeder *et al.*, 2003). Expression in all clock cells, for example, using the *Timeless (tim)-Gal4* driver, causes most flies to be arrhythmic. In contrast, *pdf-Gal4*-driven expression results in about 50% arrhythmicity for the population (Schroeder *et al.*, 2003). Consistent with these previous results, we found that *pdf-Gal4; UAS-lark* fly populations are only 42-67% rhythmic in five independent experiments, whereas *pdf-Gal4* control flies (which carry only the driver transgene) are 100% rhythmic (Table 1 and data not shown). Note also that the Rhythmicity Index (RI), a measure of the robustness of rhythmicity, was significantly lower in *pdf-Gal4; UAS-lark* flies than in *pdf-Gal4* controls and that circadian period was approximately one hour shorter than normal (Table 1; Figure 3, compare 3A to 3B). In contrast, *pdf-Gal4; UAS-lark* fly populations that only carry one functional copy of *dfmr1* (*pdf-Gal4; UAS-lark/dfmr1*) were 83–92% rhythmic in three different experiments. As shown in Figure 3 and Table 1, these flies are more similar to control *pdf-Gal4* and *pdf-Gal4; dfmr1* populations, with regard to the RI, than to *pdf-Gal4; UAS-lark* populations. However, the average circadian period was slightly short, similar to the rhythmic *pdf-Gal4; UAS-lark* flies (Table 1). These results indicate that reducing dFMRP function (*i.e*., heterozygosity for a *dfmr1* loss-of-function allele) suppresses at least one aspect of the LARK overexpression phenotype (arrhythmicity). One interpretation of these data is

that dFMRP normally promotes LARK activity *in vivo*, and therefore reducing dFMRP results in decreased LARK function and more robust rhythmicity.

In a second set of experiments, we examined flies overexpressing *dfmr1* (*pdf-Gal4; UAS-dfmr1*). We found that most were rhythmic (in 4 experiments, 68–87% rhythmic, one experiment shown in Table 1) and that circadian period was lengthened as previously reported (Dockendorff *et al.*, 2002); however, the percent rhythmicity and robustness of rhythms were slightly reduced relative to flies carrying only the *pdf-Gal4* driver (Table 1). We also found that *pdf-Gal4; UAS-lark-RNAi* flies, which express a *lark* RNAi transgene that knocks down but does not eliminate LARK within the PDF cells (Sundram and Jackson, in preparation), had normal rhythmicity (93 and 100% rhythmic in two independent experiments; one dataset shown in Table 1). In contrast, *pdf-Gal4; UAS-dfmr1/UAS-lark-RNAi* flies, that express both transgenes, were poorly rhythmic (37–47% rhythmic in 2 independent experiments; one shown in Table 1). Thus, partial loss of *lark* function enhances the overexpression phenotype of *pdf-Gal4; UAS-dfmr1* flies, which is consistent with the idea that LARK normally antagonizes dFMRP function.

The genetic interactions between *lark* and *dfmr1*, based on circadian behavior, strongly suggest that LARK and FMRP cooperate, *in vivo*, to regulate behavior. However, our previous Western blotting experiments had suggested that LARK stabilized dFMRP (see Figure 1B) whereas the behavioral genetic interactions - enhancement of dFMRP overexpression by decreased LARK and suppression of LARK overexpression by decreased dFMRP – are not in accord with such a simple model. As the behavioral results utilized the *pdf-Gal4* driver, we wished to determine how LARK and FMRP biochemically interact within PDF neurons. To examine potential protein interactions in the PDF cells, we conducted immunostaining experiments with LARK, dFMRP, and PDF antibodies using flies with increased (*pdf>uas-lark*) or decreased (*pdf>larki*) LARK levels (Supplemental Figure 2). We found that knockdown of LARK in either the large or small PDF neurons (small LN_v or large LN_v of Supp. Fig. 2) resulted in an increase in dFMRP immunosignal (Green signal) within PDF cells (Blue signal), *rather than a decrease*, although the increased signal within the small LN_vs was not statistically significant given the sample sizes in our experiments (Supp. Fig. 2B). Furthermore, overexpression of LARK in the PDF cells was associated with decreased FMRP immunosignal *rather than an increase* (Supp. Fig. 2A, B). It is clear from these experiments that LARK does not simply stabilize dFMRP in the PDF neurons and that decreases in dFMRP observed in whole animal extracts, with LARK knockdown or knockout, must reflect stabilization of the protein by LARK in non-PDF cells (and perhaps in non-neural tissues). We address the interpretation of these results in the Discussion section.

DISCUSSION

Our studies demonstrate both physical and genetic interactions between dFMRP and LARK. On the basis of circadian behavioral phenotypes, we infer that dFMRP promotes LARK function *in vivo*, whereas LARK may antagonize dFMRP function. Larvae lacking LARK or with severe deficits for the protein in all tissues have significantly decreased dFMRP levels. However, a knockdown of LARK specifically within the PDF clock neurons leads to increased dFMRP immunosignal in that cell type. We interpret these results to mean that LARK stabilizes dFMRP in certain tissues but apparently not in the PDF cells. It is expected that a knockdown of LARK and the concomitant increased dFMRP within PDF cells will genetically enhance the effects of dFMRP overexpression (the observed result for behavior). If LARK normally antagonizes dFMRP function, then decreased amounts of LARK are predicted to lead to increased dFMRP activity in addition to increasing dFMRP amount. It is difficult to rationalize the suppression of LARK overexpression that is observed in *pdf-Gal4; UAS-lark/dfmr1* flies (*i.e.*, better rhythmicity; Fig. 3) based simply on decreased dFMRP amounts. In principle, such

a decrease ought to result in worse rhythmicity unless dFMRP regulates LARK activity. Thus, we favor a model wherein LARK activity is promoted by dFMRP binding; the reduced dFMRP in *pdf-Gal4;UAS-lark/dfmr1* flies is then expected to suppress the effects of LARK overexpression. Alternative models specifying differential effects of the two RNA-binding proteins on target RNA molecules may also explain the interactions although it is premature to specify explicit biochemical models.

With respect to eye development, our results suggest that loss of dFMRP enhances a LARK-induced rough-eye phenotype, as if dFMRP normally represses LARK function in this tissue. The different genetic interactions observed for behavior versus eye development may reflect the regulation of distinct target RNAs in retinal tissue versus the PDF cell population. Alternatively, the presence of additional proteins in the LARK/dFMRP complex may modulate the activity of either or both of the RNA-binding proteins in a tissue-dependent manner. Given the plethora of functions to which RNA-binding proteins contribute in different cellular compartments – processing, export, stability, localization and translation of mRNAs (Dreyfuss et al., 2002) – which can result in promotion or repression of gene expression, it is impossible to assign meaning to the different genetic interactions in the eye versus clock cells without additional mechanistic understanding of the LARK-dFMRP interaction and knowledge about the relevant target mRNAs.

Target RNAs have been reported for dFMRP (Reeve *et al.*, 2005; Zarnescu *et al.*, 2005), although only a few of them have been validated *in vivo* by genetic analysis (Reeve *et al.*, 2005). Similarly, target RNAs were recently reported for fly LARK – this RNA-binding protein is thought to be associated with as many as 200 different RNAs *in vivo* (Huang *et al.*, 2007). For both dFMRP and LARK, a number of putative targets are known to have circadian functions or to display circadian changes in abundance. In addition, there are at least 12 targets that overlap from the two independent studies (Y. Huang, and F.R. Jackson, unpublished results; see Methods for data mining procedures); it is possible that these RNAs represent common targets that are modulated by both proteins. Finally, as dFMRP is known to be in a complex with Lgl (Zarnescu *et al.*, 2005), and interacts with it to regulate eye development, it might be the case that all three proteins – LARK, dFMRP, and Lgl execute certain functions together, *in vivo*, perhaps by regulating common targets. Presumably, certain targets encode proteins that function as elements of the clock output pathways regulating behavior, whereas others may mediate functions relevant for eye development.

Of related interest, it has been shown that mammalian and fly FMRP are associated with Argonaute (Ago) proteins and micro-RNAs (miRNAs) *in vivo* and that Ago1 is necessary for FMRP function *in vivo* in *Drosophila* (Caudy *et al.*, 2002; Jin and Warren, 2003). More recently, it was shown that human RBM4 (hLARK) is associated with Ago/FMRP/miRNA complexes and that knockdown of RBM4 in human cells results in altered miRNA-mediated gene regulation for several different mRNA targets (Hock *et al.*, 2007). Another recent study demonstrated that a significant number of fly LARK RNA targets contain consensus binding sites for miRNAs, suggesting that the fly protein may also be required for miRNA-mediated gene regulation (Huang *et al.*, 2007). Thus, dFMRP and LARK may function together with miRNAs in the regulation of certain RNA targets.

How might LARK and dFMRP function together to regulate development or behavior? Although the proteins are predominantly located in distinct intracellular compartments - LARK in the nucleus and dFMRP in the cytoplasm – both vertebrate homologues are known to shuttle between the two compartments (Feng *et al.*, 1997b; Lai *et al.*, 2003), and they may function together in the cytoplasm to regulate the translation of target RNAs. Indeed, cell-based studies have recently suggested that mouse LARK (mLARK or Rbm4) becomes phosphorylated, in a MAPK-dependent manner, in response to cell stress, and then shuttles into the cytoplasm to

regulate the IRES-dependent translation of target RNAs (Lin *et al.*, 2007). mLARK can bind to a sequence within the 3' untranslated region of the mouse *Per1* mRNA (a known clock factor), and it has been suggested that the observed rhythm in LARK abundance within the mouse suprachiasmatic nuclei (SCN) serves to modulate the translation and circadian expression of PER1 protein (Kojima *et al.*, 2007). Of interest, mLARK overexpression in cell-based assays increases PER level and slightly lengthens circadian period whereas increased fLARK expression, *in vivo*, seems to shorten period (Table 1). We do not understand this species difference but the fLARK result is consistent with a promotion of PER expression, which in flies is predicted to shorten circadian period (Baylies et al., 1987). Nonetheless, our results suggest that LARK and dFMRP cooperate within certain cell types to post-transcriptionally regulate target RNAs that are relevant for circadian and other biological functions.

A role for *Fmr1* in mammalian circadian behavior has recently been established (Zhang *et al.*, 2008). Mice that lack *Fmr1* exhibit a short circadian period in free-running conditions, while those lacking both *Fmr1* and *Fxr2* display arrhythmic locomotor activity as measured by wheel running during light:dark conditions. This anomaly appears to be due to clock output, since the suprachiasmatic nuclei of double knockout animals appear to function normally. These data underscore the relevance of the *Fmr/Fxr* gene family to circadian control. The finding that LARK and dFMRP interact in the fly suggests that mLARK and FMRP/FXR2P interactions may also be relevant in the mouse in exerting circadian control.

Finally, the importance of sleep in learning and memory consolidation, and also in the generation/formation of new memory (Walker and Stickgold, 2004; Stickgold, 2005; Yoo *et al.*, 2007) suggests that the abnormal circadian clock and sleep patterns in FXS patients could contribute to their cognitive impairment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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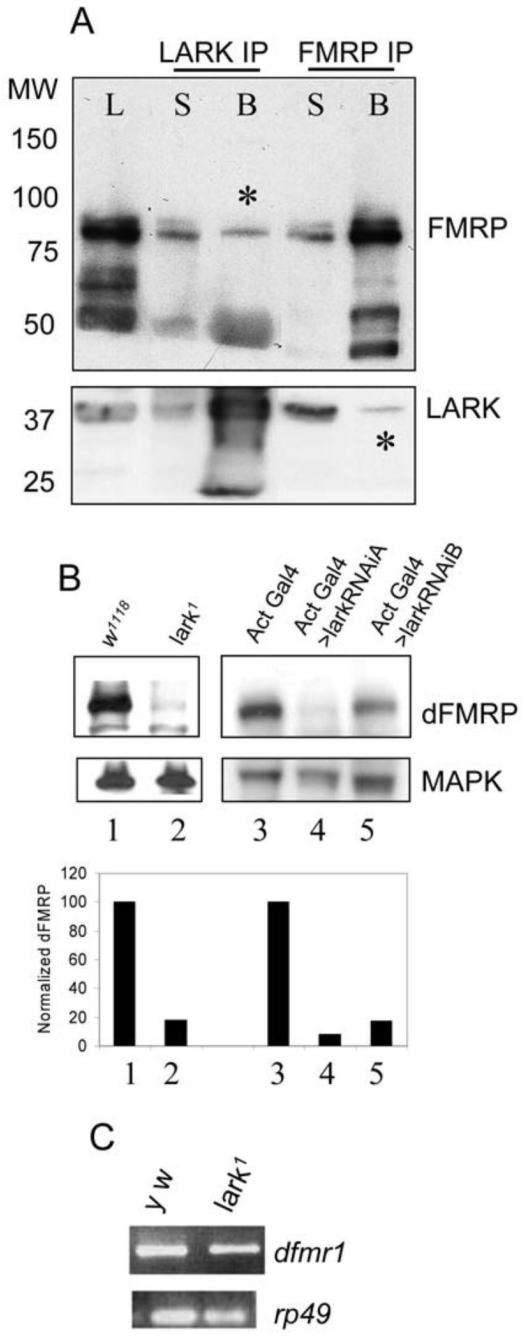

Figure 1. Biochemical and genetic interactions between dFMRP and LARK

A. Co-immunoprecipitation (CO-IP) was performed on fly head lysates using either anti-dFMRP or anti-LARK antibody. The immunoprecipitates were then analyzed by immunoblotting using the same antibodies. Asterisks indicate dFMRP (middle lane) or LARK (far right lane) that was precipitated by antibody against the other protein. L, lysate; B, beads plus antibody; S, supernatant after IP. **B.** dFMRP levels are reduced in larvae missing LARK and in pupae with reduced LARK amounts (lanes 2, 4 and 5), MAPK was used as a control for protein loading. **C.** *dfmr1* RNA levels are comparable in wild-type flies and those with reduced LARK amount. *rp49* was used as control for RNA loading.

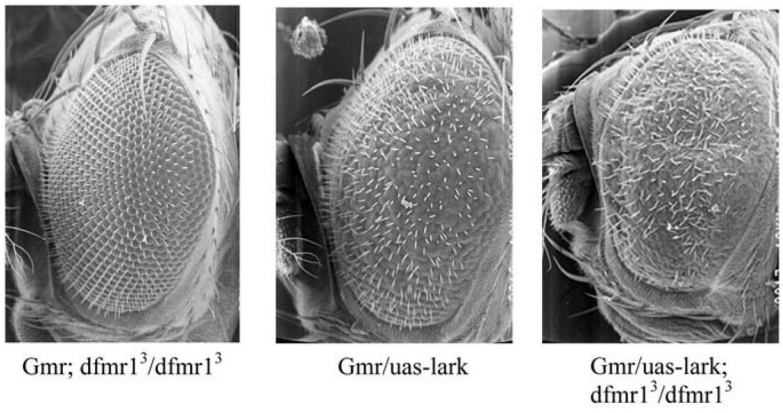

Figure 2. The eye phenotype resulting from LARK overexpression is enhanced by the elimination of dFMRP

A. *dfmr1* null fly, displaying a wild-type eye phenotype. **B.** *gmr-Gal4; UAS-lark* fly. High level expression of LARK in the eye, using *gmr-Gal4*, causes a disorganized eye phenotype. Note the fused ommatidia and general disruption of interommatidial bristles. **C.** *gmr-Gal4/ UAS-lark; dfmr1/dfmr1* fly with severe eye phenotype.

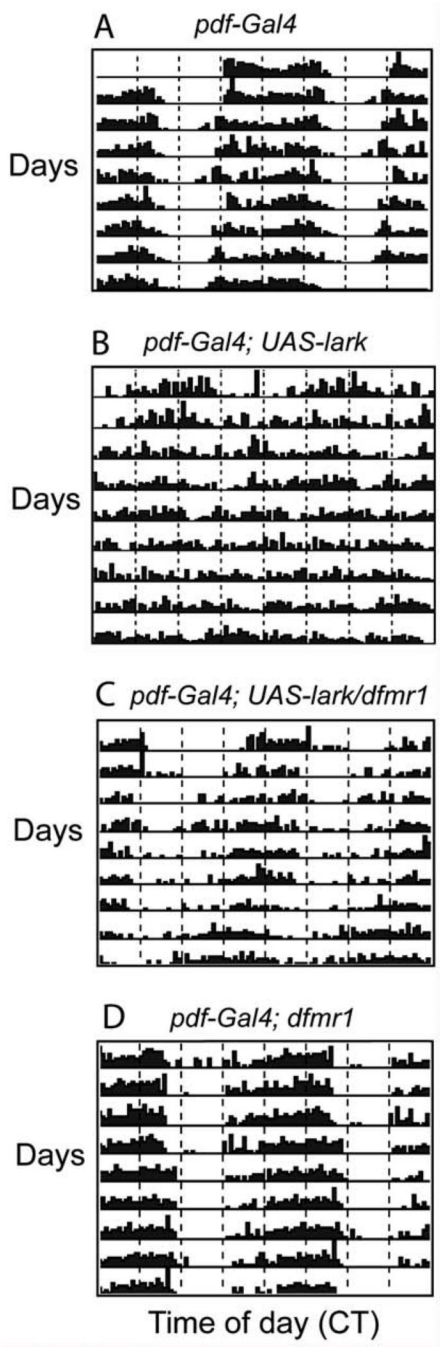

Figure 3. Reducing *dfmr1* expression suppresses the rhythm phenotype of flies overexpressing LARK

A. Control *pdf-Gal4* fly with a wild-type locomotor activity rhythm. **B.** Overexpression of LARK causes arrhythmicity. **C.** Heterozygosity for a *dfmr1* null mutation ameliorates the rhythm phenotype of flies overexpressing LARK. **D.** *pdf-Gal4; dfmr1* fly (heterozygous for *dfmr1* null) with a wild-type activity rhythm.

Table 1

Genetic interactions between *dfmr* and *lark* for circadian behavior.

Genotype	n	% Rhythmic	RI ± SEM	Period ± SEM (h)
pdf-Gal4	17	100	0.43 ± 0.03	24.0 ± 0.07 (n=17)
pdf-Gal4; UAS-lark *	26	50	0.24 ± 0.02	23.2 ± 0.17 (n=13)
pdf-Gal4;UAS-lark/dfmr1 *	23	83	0.40 ± 0.03$^{\alpha}$	23.2 ± 0.10 (n=19)
pdf-Gal4; dfmr1/+ *	23	96	0.51 ± 0.03	24.2 ± 0.04 (n=22)
pdf-Gal4; UAS-lark-RNAi$^{\varphi}$	28	93	0.39 ± 0.03	24.6 ± 0.12 (n=26)
pdf-Gal4; UAS-dfmr$^{\varphi}$	23	70	0.19 ± 0.03	26.2 ± 0.42 (n=16)
pdf-Gal4; UAS-dfmr/UAS-lark-RNAi$^{\varphi}$	22	37$^{\lambda}$	0.13 ± 0.02	23.7 ± 0.29 (n=8)

* Populations assessed behaviorally in the same experiment.

$^{\varphi}$ Populations assessed behaviorally in the same experiment.

$^{\alpha}$ Different from *pdf-Gal4; UAS-lark*, $p < 0.001$ and from *pdf-Gal4; dfmr1/+*, $p<0.05$, ANOVA with Bonferroni's multiple comparison test.

$^{\lambda}$ Different from *pdf-Gal4; UAS-lark-RNAi* ($p < 0.01$) and *pdf-Gal4; UAS-dfmr* ($p < 0.02$)